

Venoms to drugs

Toxic fingers pick ASIC's pocket: chemical synthesis, 3D structure and ASIC binding site of mambalgin-2**

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Abstract: Mambalgins (Ma) are a novel class of snake venom components that have potent analgesic effects mediated through inhibition of acid-sensing ion channels (ASICs). We report the chemical synthesis of the 57-residue polypeptide Ma-2 using a combination of solid phase peptide synthesis and native chemical ligation. The structure of the synthetic toxin determined using homonuclear NMR revealed an unusual three-finger toxin fold reminiscent of functionally unrelated snake toxins. Electrophysiological analysis of Ma-2 on wild-type and mutant ASIC1a receptors allowed us to identify α -helix 5 of the channel, which borders on the functionally critical acidic pocket, as a major part of the Ma-2 binding site. This region is also crucial for interaction of ASIC1a with the spider toxin PcTx1, suggesting that the binding sites for these toxins substantially overlap. This work lays the foundation for SAR studies and further development of this promising analgesic peptide.

Animal venoms are essentially large combinatorial libraries of bioactive molecules, which hold great promise as diagnostic tools and in treatment of human diseases.^[1] The vast majority of these compounds are disulfide-rich polypeptides of 10–80 amino acid residues that adopt highly-ordered 3D structures and potentially modulate the activity of specific classes of membrane proteins such as ion channels, receptors and transporters. Mambalgins are a recently discovered class of peptides isolated from the venom of black mamba snakes. They are comprised of 57 residues cross-braced by four disulfide bridges.^[2] The mambalgins are potent blockers of acid-sensing ion channels (ASICs) and they show potent central and peripheral analgesic effects in animal models without the side effects associated with traditional opioid drugs.^[2a] In addition to a role in pain perception, this family of proton-gated cation channels has been implicated in neurodegeneration, fear and anxiety-related behaviour, brain tumour growth and sensory transduction.^[3]

Based on the number and location of the cysteine residues, and the length of the inter-cysteine sequences ('loops'), the mambalgins were proposed to adopt a 'three-finger toxin' (TFT) fold reminiscent of the functionally unrelated short-chain neurotoxins commonly found in snake venom.^[4] However, the disulfide connectivity has not been determined experimentally, the inter-cysteine loops show very limited similarity to known TFTs, and the mambalgins possess activity not previously observed for TFTs, thereby raising the possibility that the mambalgins have a unique 3D fold (see Supporting Information, SI). To address this possibility and to establish a robust platform for detailed structure-activity relationship studies, we report here the first efficient chemical synthesis and 3D structure determination of a member of the mambalgin family (Ma-2).

We set out to chemically synthesize the entire 57-residue Ma-2 by step-wise Fmoc solid-phase peptide synthesis (SPPS). Despite several attempts using optimized protocols we were unable to produce even trace amounts of the target molecule. Detailed MALDI analysis indicated complete termination of chain extension around residue Lys31.

To circumvent these problems, we turned to Boc chemistry employing a peptide fragment ligation approach via Kent's native chemical ligation (Scheme 1).^[5] This approach has been instrumental in enabling access to unrelated but similarly complex venom-derived disulfide-rich molecules.^[6] The synthesis strategy for Ma-2 employed three segments with Cys19 and Cys37 serving as ligation sites. Temporary protection of Cys19 was realized by substituting this residue with 1,3-thiazolidine-4-carboxylic acid.^[7] Peptide thioesters corresponding to Ma-2[1–18] and Ma-2[19–36] were assembled by optimized Boc-SPPS, whereas Ma-2[37–57] was produced by Fmoc chemistry.^[8] All peptide segments were obtained in high yield and purity and were subsequently joined using native chemical ligation in consecutive one-pot fashion as described previously (see SI for experimental details).^[6c; 7; 9] The full-length polypeptide was obtained in good yield after HPLC purification (33%). Although peptides of this size are generally considered amenable to optimized stepwise SPPS,^[10] our experience suggests that fragment-based assembly may deliver superior results.

In vitro folding of the polypeptide chain and concomitant formation of the four disulfide bridges required optimization. Because of the large number of potential disulfide isomers (105), optimization was guided by HPLC analysis and activity testing of isolated fractions on rat (r) ASIC1a (see below). Folding was most efficient in 100 mM Tris, 500 mM guanidine HCl, pH 8.0 containing a redox system of 8 mM reduced glutathione (GSH) and 1 mM oxidized glutathione (GSSG). Under these conditions, the reaction reached equilibrium in 24 h and the correctly folded Ma-2 was isolated in high purity and acceptable yield after HPLC purification (11%). The activity of the synthetic material was assessed using two-electrode voltage-clamp electrophysiology on *Xenopus laevis* oocytes heterologously expressing homomeric rASIC1a and rASIC1b (Figure 2a). Synthetic Ma-2 caused a concentration-dependent inhibition with an IC_{50} of 21 ± 6 nM for rASIC1a and 103 ± 13 nM for rASIC1b ($n = 5$). Both values are slightly lower (indicating higher potency) than the values previously reported for native Ma-2 isolated from snake venom (rASIC1a: 55 nM; rASIC1b: 192 nM),^[2a] underscoring the high quality of the synthetic material.

The 3D structure of synthetic Ma-2 was determined using homo-nuclear 2D NMR spectroscopy (see SI for details). The solution structure of Ma-2 was calculated from 1003 interproton-distance and dihedral-angle restraints using CYANA 3.0^[11] and refined using CNS.^[12] Analysis of the secondary H α chemical shifts compared to those found in random coil peptides revealed several stretches of positive chemical shifts indicative of β -sheet formation (SI Figure 3).^[13] Several residues, most notably Gly36, Arg54 and Arg57, experienced unusually large chemical shift differences most likely due to aromatic ring current effects due to proximity to Tyr20 and Phe4 (SI Figure 3). Statistical analysis of the inferred disulfide bond connectivity in the resultant structure was performed using PADLOC^[14] and was in agreement with Cys3–Cys19, Cys12–Cys37, Cys41–Cys49 and Cys50–Cys55.

An ensemble of the 20 lowest energy structures of Ma-2 is shown in Figure 1a. Overall, the structure is well defined with a fold reminiscent of TFTs (SI Figure 4). The core of the molecule is stabilized by four disulfide bridges with the same connectivity as in other short-chain neurotoxins. Hydrogen-deuterium exchange TOCSY experiments indicated several slow exchanging amide protons. In particular, backbone amide protons of residues 10, 18, 20 and 52 were still detectable after 21 h indicative of strong hydrogen bonding in the core of the molecule. Three loops protrude from this core (Figure 1b). The first (residues 4–11) and the third (residues 42–48) ‘finger’ are structurally well defined, although they are shorter than in other TFTs (SI Figure 4). In contrast, the longer middle finger (residues 20–36) is disordered from residues Thr23 to Ile33 in agreement with TALOS-N predictions that they are flexible.^[15] An extensive hydrogen-bonding network between finger II and III gives rise to a three-pleated antiparallel β -sheet. Finger I forms a separate antiparallel β -sheet that is structurally constrained by disulfides 3–19 and 12–37. This arrangement of secondary structural elements is characteristic for short-chain TFTs (SI Figure 4). Despite the similar core, the structure of Ma-2 stands out among TFTs due to its short first and third fingers and elongated middle finger.

The peptide surface contains several clusters of basic or acidic residues (Figure 2b): 1) a large, positively charged cluster located in the first finger and the core domain; 2) Glu43 and Glu45 in the third finger and 3) Arg 28 and Lys31 located at the tip of the middle finger. The latter is adjacent to a hydrophobic patch formed by Met25, Phe27, Leu30, Leu32 and Leu34. This arrangement is reminiscent of psalmotoxin 1 (PcTx1), a potent modulator of ASIC1 isolated from tarantula venom (Figure 2b).^[16] PcTx1 binds to the acidic pocket at the interface between ASIC1 subunits (Figure 2c) that serves as a putative proton-sensing site.^[17] In doing so, PcTx1 alters the proton sensitivity of the channel and stabilizes the desensitized state. Interestingly, mambalgins have been shown to decrease ASIC sensitivity to protons suggesting that they might also act as gating modifiers and directly or indirectly interfere with the proton sensing mechanism.^[2a]

Based on the structural and potentially mechanistic similarities between PcTx1 and Ma-2 we hypothesized that these toxins might target the same site on ASIC1. To test this hypothesis we generated rASIC1a carrying an F350A mutation. This residue, which is highly conserved in ASIC1, is located on α -helix 5 (the 'thumb region'), just outside the acidic pocket (Figure 2c). An F350L mutation was previously shown to essentially abolish PcTx1 action on human ASIC1a.^[18] The F350A mutant channel functioned similarly to wild type rASIC1a albeit with an acidic shift in the pH sensitivity of steady-state desensitisation and activation ($\Delta\text{pH}_{0.5} = -0.17$ and -0.37 , respectively, see SI Figure 5). This is not surprising as Phe350 lies adjacent to the proton-sensing residue Asp349, and the F350L mutation in hASIC1a resulted in a similar ~ 0.4 pH unit acidic shift in the $\text{pH}_{0.5}$ of activation.^[18] The mutant channel was dramatically less sensitive to Ma-2 than wild-type rASIC1a ($\text{IC}_{50} > 1 \mu\text{M}$, Figure 2a). This more than 50-fold drop in toxin efficacy indicates that Phe350 plays a key role in sensitizing ASIC1a channels towards Ma-2 as it does towards PcTx1.^[18]

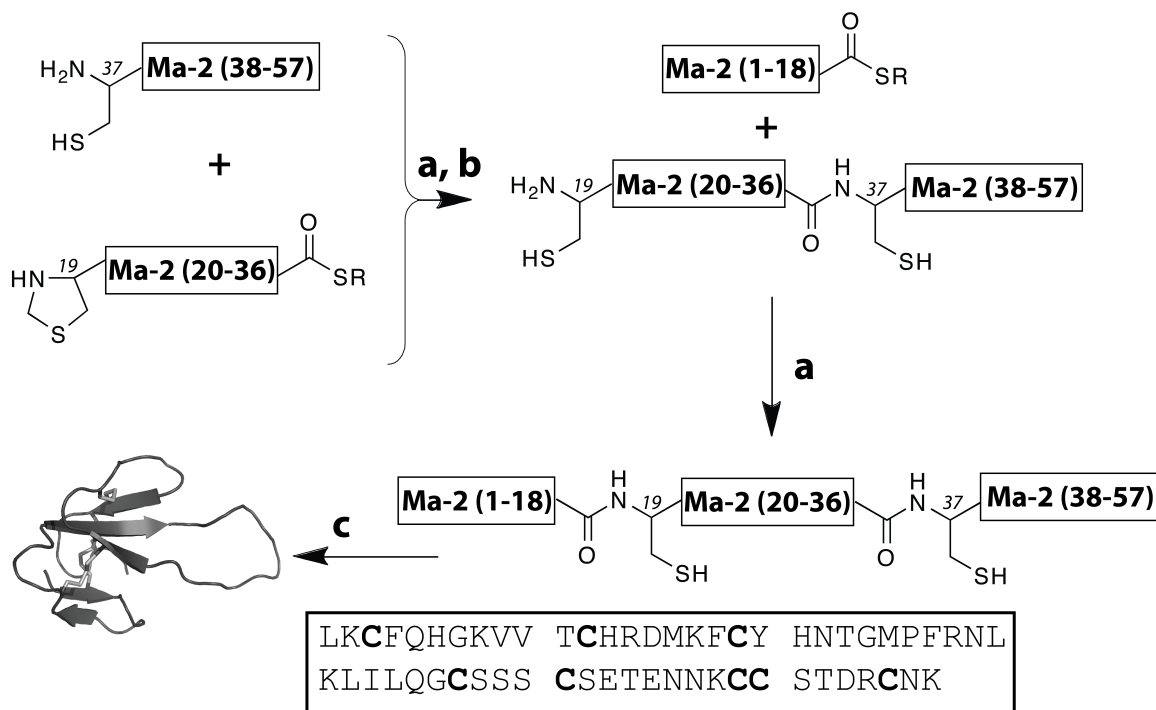
PcTx1 is an equipotent inhibitor of ASIC1a homomers and ASIC1a/2b heteromers^[18] and potentiates ASIC1b homomers,^[19] whereas the mambalgins inhibit both ASIC1a and ASIC1b homomers and ASIC1a heteromers.^[2a] Thus it seems that both peptides can interact, with similar relative efficacy, at the same range of ASIC subtypes.^[19] In light of their similar subtype selectivity and the fact that both toxins require F350 for functional interaction with ASIC1, it seems likely that they have similar binding sites, albeit with varied functional consequences (PcTx1 can potentiate ASICs,^[19] whereas mambalgins so far have only been reported to cause inhibition).

In summary, we demonstrated efficient chemical synthesis of the analgesic venom peptide Ma-2. We anticipate that our synthetic approach will not only enable detailed structure-activity studies of Ma-2 but that it will also be applicable to other family members due to the high degree of sequence conservation within the mambalgins. We provide the first experimental proof that mambalgins adopt a TFT fold. The mambalgins therefore represent another example of the evolutionary divergence of the TFT fold in order to functionally diversify the toxin repertoire of venomous snakes.^[20] Finally, our functional data suggest that the mambalgins bind in close proximity to the acidic pocket of ASIC channels in a

manner very similar to PcTx1, most likely by insertion of one of their protruding 'fingers'.

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Scheme 1. Synthesis of Ma-2 by native chemical ligation of three peptide segments. (a) native chemical ligation: 6 M GdmHCl, 0.2 M sodium phosphate, 50 mM 4-mercaptophenylacetic acid, 50 mM Tris (2-carboxyethyl) phosphine HCl, pH 7.0; (b) Thz→Cys conversion: 0.3 M methoxyamine HCl, pH 4.0; (c) folding and disulfide formation: 100 mM Tris, 500 mM GdmHCl, 8 mM GSH, 1 mM GSSG, pH 8.0.

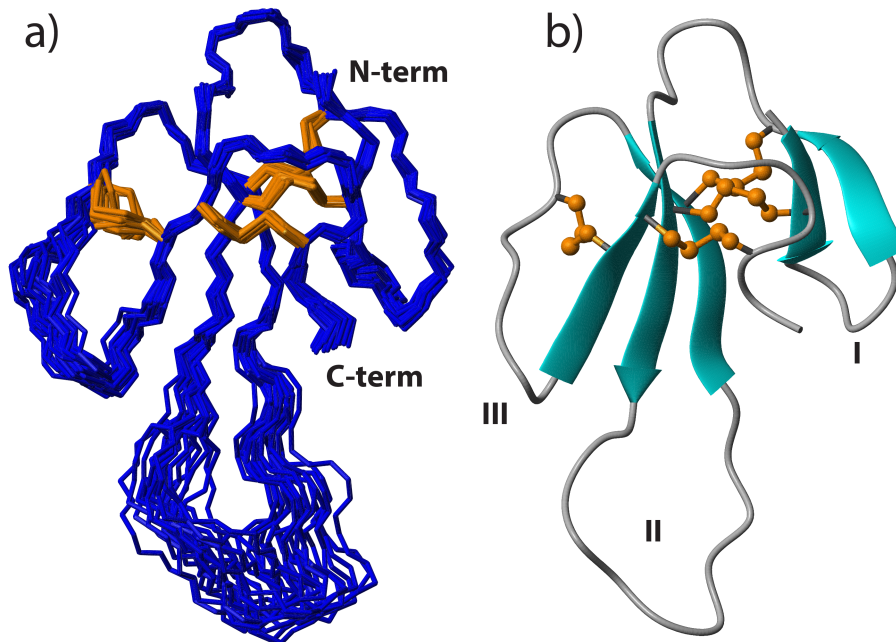


Figure 1: Structure of Ma-2. (a) Ensemble of 20 lowest-energy Ma-2 structures (PDB ID: 2MFA) superimposed over the backbone of the well-ordered residues 1–22, 34–41 and 47–57. The N- and the C-termini are labelled and the four disulfide bonds are shown in orange. (b) Ribbon representation of the lowest energy Ma-2 structure showing three fingers protruding from a disulfide-stabilized core. Note the two β -sheets; the antiparallel triple-stranded β -sheet includes fingers II and III while the antiparallel double-stranded β -sheet encompasses finger I.

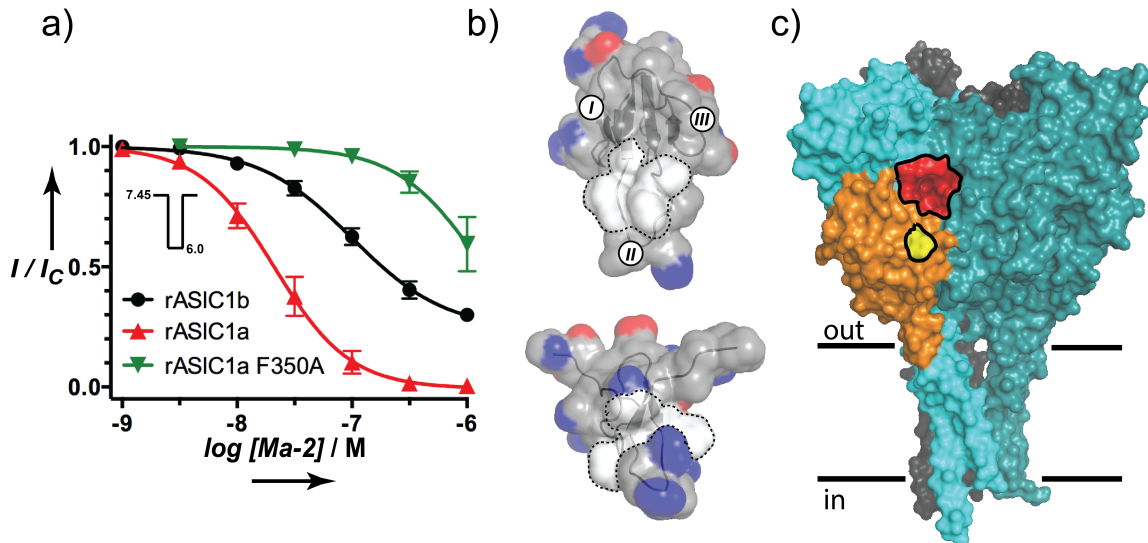
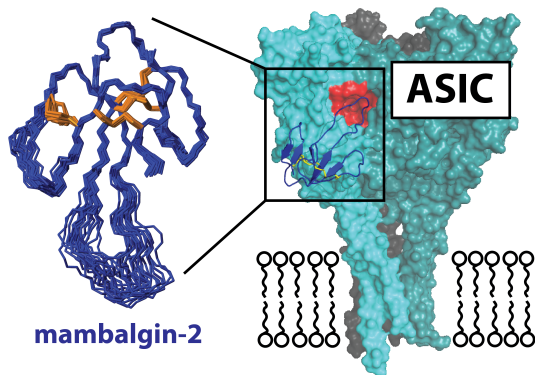


Figure 2: Structural and functional characterization of synthetic Ma-2. (a) Concentration-response curves for inhibition by synthetic Ma-2 of rASIC1a, rASIC1b, or an F350A mutant of rASIC1a (all $n = 5$). I/I_c : test current/control current. The inset shows the pH jump protocol used for channel activation. (b) Surface charge distribution of Ma-2 (upper panel) and PxDc1 (PDB ID: 2KNI, lower panel).^[21] The hydrophobic patch is coloured white and Ma-2 fingers are labelled I-III. (c) Structure of chicken ASIC1 (PDB ID: 4FZ0).^[17a] The three subunits that form the functional channel are shown in different shades of grey and the locations of the acidic pocket (red), Phe350 (yellow) and the thumb region (orange) are indicated.



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Mambalgin fingers pick ASIC's pocket: Efficient chemical synthesis of mambalgin-2 using native chemical ligation permitted the first structure determination of a member of this family of analgesic snake toxins. Electrophysiological analysis suggests that Ma-2 binds near the acidic pocket on ASIC channels.